

# Peptides removing in enzymatic membrane bioreactor

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## Abstract

Applicability of thermolysin — one of proteolytic enzymes, in catalyzing the process of hydrolysis of a mixture of polypeptides being a waste product of dairy industry was discussed in the study. Process temperature (50°C) was selected and kinetics of the process involving a native enzyme was determined. The Michaelis–Menten equation constants are  $K_M = 34.29 \text{ g L}^{-1}$ ,  $k_3 = 14,285.7 \text{ g}_{\text{pr.}} \cdot \text{g}_{\text{enz.}}^{-1} \text{ h}^{-1}$ .

In order to reuse the catalyst a possibility of its immobilization by adsorption on the surface of a cellulose nitrate membrane was investigated. Efficiency and stability of binding and activity of the adsorbed enzyme towards a mixture of polypeptides were determined. In the applied range of substrate the model of first-order kinetics can be applied and the value of the constant is  $k_r = 6615.4 \text{ h}^{-1}$ .

Due to low stability of the immobilized preparation results most probably from a gradual detachment of the enzyme from the membrane it was proposed to carry out the continuous process in an integrated system which would combine the advantages of an agitated reactor (high enzyme activity and stability) with those of a packed bed (high enzyme concentration in small volume, low substrate concentration at the exit). The presence of a properly selected membrane causes that enzyme molecules cannot leave the bioreactor, they can only transfer between the packed zone on the membrane surface and the zone of complete mixing in the reactor volume. So, the membrane will have two functions: a separator for the native enzyme and a matrix for enzyme immobilization and will be created dynamically.

*Keywords:* Peptide hydrolysis; Membrane reactor; Enzyme adsorption; Catalytic membrane; Dynamic membrane

## 1. Introduction

Wastewaters generated by dairy industry contain very large quantities of proteins and

short-chain peptides. They can be treated by microbiological methods because they are a very rich nutrient which contains both carbon and nitrogen sources. However, microbiological treatment of these wastewaters causes a significant growth of biomass of different microbial

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species which becomes a pollutant itself. The biodegradation processes are often accompanied by protein production by these microorganisms [1–3], which causes that protein mass in the wastewater is not eliminated to a significant degree. Therefore, in the case of this pollutant type the use of proteolytic enzymes seems much more promising.

In the enzymatic processes carried out at an industrial scale, immobilized preparations are applied for operational and economic reasons. Thus, the existing methods of immobilization are improved and new techniques are searched [4–6]. Among many methods, separation on membranes finds increasing applications because membrane properties offer broad and interesting possibilities of effective enzymatic reactions.

In the membrane reactors, the processes can be carried out

- in the presence of a native enzyme, with complete mixing and the separation membrane holding up a biocatalyst in the reaction zone [7,8],
- with an enzyme deposited on the membrane, in plug flow conditions [9,10].

Immobilization of an enzyme in the membrane reactor by maintaining it in its native form in the reactor volume is an efficient technique which is easy to perform. Variety of commercially available membranes, both for pressure processes and for diffusion, offers large possibilities for carrying out this process. A disadvantage of this method of immobilization is a relatively quick enzyme inactivation in the most cases of enzymes which can be reduced by adding fresh portions of the catalyst (not necessarily of high purity) during the process [11]. Low enzyme concentration in the reaction mixture enables multiple repetition of this procedure without risking a deterioration of rheological properties of the system. This affects naturally the system economics.

Immobilization by fixing the enzyme on a carrier (including a membrane) can be obtained by

- adsorption on the carrier surface [12–14],
- chemical binding of a protein particle with the carrier surface [15–17],
- inclusion in the carrier volume available for the reagents [18–20].

Among these methods the cheapest is adsorption. Moreover, preparations obtained in this way are often characterized by a sufficiently good stability [21].

The use of thermolysin in the native form and immobilized on the membrane to decompose polypeptides coming from partial hydrolysis of casein is considered in this study.

## 2. Materials and methods

### 2.1. Materials

The enzyme: thermolysin (Protease type X from *Bacillus thermoproteolyticus*) was supplied by Sigma (USA). Casein Hammersten was purchased from Loba Feinchemie (Austria), other reagents from Sigma (USA).

Membranes — flat, polymeric-cellulose nitrate, asymmetric, pores diameter 0.02  $\mu\text{m}$  from Euro-Sep Ltd. (Poland).

Equipment — membrane plate modules with active area of 4.9  $\text{cm}^2$ , thermostated mixing reactors, ultrafiltration cell by Millipore (USA), gear (pressure) pump supplied by Cole-Parmer (USA), spectrophotometer UV-Vis Helios  $\alpha$  by ThermoSpectronic (UK).

### 2.2. Analytical methods

The concentration of enzyme and casein was determined by the Lowry's method [22].

The reaction substrate (a mixture of polypeptides) was obtained from partial hydrolysis of milk protein (casein), during which the process was controlled by means of electrophoresis. Partial hydrolyzate was centrifuged (10 min, 4000 rpm),

so that not hydrolyzed protein residues remained in the deposit. The supernatant containing polypeptides was used as a substrate at next, main stages of the research. Polypeptides concentration in the supernatant was calculated on the base of mass balance between the initial mass of casein and the dry casein in the deposit.

The number of peptide bonds released in the reaction was determined by the method described in literature [23] which is based on the reaction of *o*-phthaldialdehyde with free  $-\text{NH}_2$  groups in an alkaline medium, in the presence of a reducing reagent (e.g. 2-mercaptoethanol). A procedure concordant with [23] was used and the time of analysis was set to be 10 min. For each substrate concentration the initial and final absorbency was established (when the number of free amino groups was not increasing) and a difference of these values was assumed to correspond to  $\alpha = 1$  at a given initial substrate concentration. This relation is shown in Fig. 1.

### 2.3. Process temperature selection

The effect of temperature on an enzyme activity and stability was tested in a broad range of temperatures from 20 to 80°C. The enzyme concentration in the reaction systems was 0.10 mg L<sup>-1</sup> and initial peptide concentration was 10.0 g L<sup>-1</sup>.

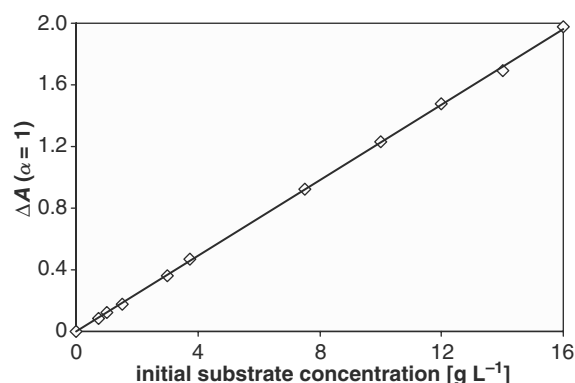


Fig. 1. Dependence of the maximal value  $\Delta A$  (340 nm) on the initial substrate concentration.

The process of hydrolysis was performed in 100 mL thermostated mixing reactors. The enzyme activity at 39°C was assumed as 100% activity.

Thermostability was determined after incubation by 3 h in a 0.1 M phosphate buffer, pH 7.5 at given temperature (ranging from 8 to 100°C) and then making a standard test at the temperature 39°C. Stability of the enzyme was given as the percent of the initial catalytic activity determined at the temperature 39°C prior to incubation.

### 2.4. Kinetics of hydrolysis with a native enzyme

The process of polypeptide hydrolysis in the presence of a native enzyme was carried out in thermostated (50°C) agitated reactors, at substrate concentration ranging from 2.4 to 16.2 g L<sup>-1</sup> and enzyme concentration 0.1–0.3 mg L<sup>-1</sup>. The substrate (a mixture of polypeptides) was obtained from partial hydrolysis of milk protein (casein). At time intervals samples were taken to analyze the number of free amino groups (Section 2.2), whose development corresponded to the hydrolysis of subsequent bonds in the peptide chain. From the difference of adsorbency of the initial and instantaneous sample an instantaneous  $\Delta A$  was determined, which in turn was compared to  $\Delta A$  corresponding to  $\alpha = 1$ , at a given initial substrate concentration (Fig. 1). It was assumed that when  $\alpha = 1$  the product concentration (oligopeptides) was equal to the initial concentration of the substrate (polypeptides).

### 2.5. Enzyme immobilization: kinetics of hydrolysis with an immobilized enzyme

The enzyme was immobilized in ultrafiltration cells which contained a cellulose nitrate membrane with the surface area 4.9 cm<sup>2</sup> adding 8.0 mL of the enzyme solution at a known concentration (in the range 0.221–0.867 g L<sup>-1</sup>) and pressure 0.04 MPa in the dead-end system. The

permeate was recycled twice and the procedure was repeated. The amount of enzyme deposited in this way was calculated from mass balance prepared from the solutions before and after immobilization by Lowry's method. The membranes were kept at 8°C in fresh buffer solution. After 18 h incubation the mass of weakly bounded protein was measured by Lowry's method and then the membranes were used to analyze their catalytic activity.

Activity and stability of the immobilized preparation were determined in the system with a membrane module in which the membrane with immobilized enzyme was fixed and using a pressure pump at flow rate 18.7 L h<sup>-1</sup>, pressure 0.04 MPa the substrate solution (0.03 L, concentration in the range 1.77–14.3 g L<sup>-1</sup>) of temperature 50°C was supplied to the module in the dead-end system. The permeate was recirculated to the substrate tank, so the solution was circulating constantly on the membrane. At certain time intervals samples for free amino groups analysis were taken from the tank.

### 3. Results

#### 3.1. Process temperature selection

The effect of temperature on thermolysin catalytic properties is illustrated in Fig. 2.

The enzyme has maximum activity at high temperatures (50–80°C) and preserves relatively good stability up to the temperature of 60°C, which, when exceeded, causes an abrupt increase of the enzyme inactivation.

Taking into account the above relations, for further studies the temperature 50°C was chosen. Under this condition the half-live time of the catalytic activity was determined and described according to the first-order kinetics of the inactivation reaction [24]. This value was estimated by 132 h.

#### 3.2. Kinetics of hydrolysis with a native enzyme

As it was observed the range of enzyme concentration in which the hydrolysis rate was a linear function of enzyme concentration is very narrow (only to 0.3 mg L<sup>-1</sup>) thus the reaction kinetics was determined in three series by the enzyme concentrations equal to 0.1, 0.2 and 0.3 mg L<sup>-1</sup>. The initial substrate concentration was changed in the range from 2.4 to 16.2 g L<sup>-1</sup> and the reaction rate by the substrate conversion in initial condition ( $\alpha < 0.1$ ) was estimated.

Dependence of the reaction rate on substrate concentration calculated into catalyst concentration 1 g L<sup>-1</sup> is shown in Fig. 3. This is a typical relation which can be described by the

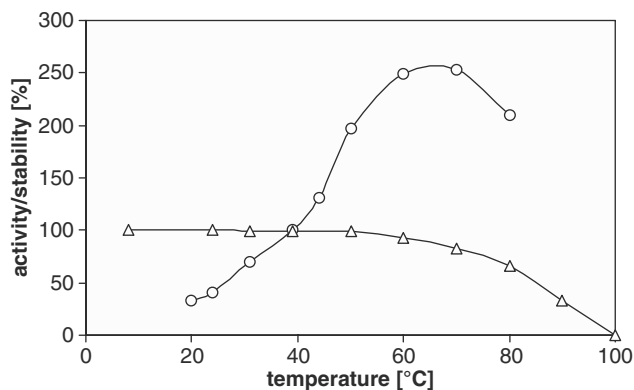


Fig. 2. Influence of temperature on activity (o) and stability (Δ) of thermolysin ( $c_e = 0.125$  mg L<sup>-1</sup>,  $c_{s,0} = 10$  g L<sup>-1</sup>).

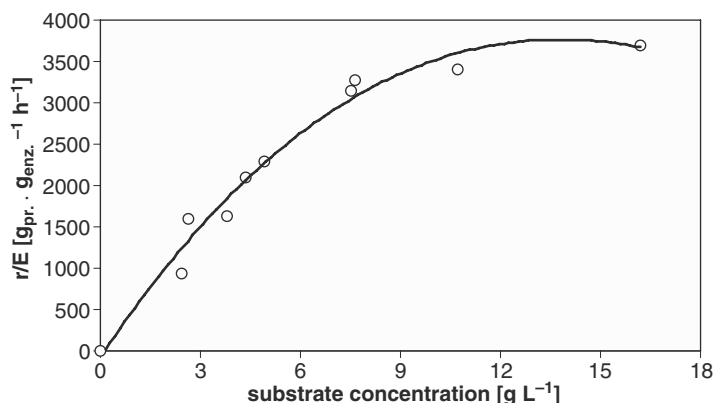


Fig. 3. Dependence of the reaction rate on the substrate concentration in reaction carried out by thermolysin in a native form.

Michaelis–Menten equation. From the Lineweaver–Burk equation [25] constants:  $K_M = 34.29 \text{ g L}^{-1}$ ,  $k_3 = 14,285.7 \text{ g pr.} \cdot \text{g enz.}^{-1} \text{ h}^{-1}$  were estimated.

### 3.3. Enzyme immobilization

The immobilization procedure was performed at different enzyme concentrations in the initial solution ranging from 0.221 to 0.867  $\text{g L}^{-1}$ . It was observed that in all cases the degree of immobilization was similar ca. 70%. After an incubation lasting for 18 h at 8°C, weakly bounded protein in water solution was detached, so finally the immobilization efficiency was 53–57.7% (Table 1).

Binding stability in the reaction conditions (Fig. 4) was determined observing activity

decrease of the preparation in time. High the decrease of activity of the immobilized preparation was observed. Half-life time of the preparation activity was determined to be only 5 h. It is rather impossible that so important change in enzyme properties towards good stability of native form (Section 3.1) has haven place. It is more probable that the decrease of activity of the immobilized preparation results from a gradual detachment of the enzyme from the membrane by the successive application the new substrate solutions during kinetics experiments.

This low stability of thermolysin adsorption to the applied membrane causes difficulties with direct application such preparation.

Table 1  
Efficiency of immobilization process

Enzyme mass in solution before immobilization (mg)	Enzyme mass in solution after immobilization (mg)	Initial immobilization efficiency (%)	Enzyme mass in solution after incubation (18 h, 8°C) (mg)	Enzyme mass immobilized on membrane ( $A = 4.9 \text{ cm}^2$ ) (mg)	Final immobilization efficiency (%)
6.938	2.220	68.00	0.902	3.816	55.00
5.024	1.497	70.20	0.863	2.664	53.02
3.573	1.089	69.52	0.50	1.977	55.33
1.768	0.530	70.02	0.218	1.020	57.69

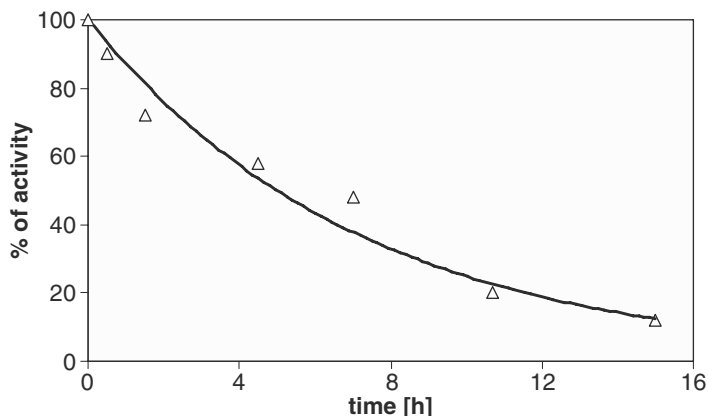


Fig. 4. Stability of the immobilized preparation.

### 3.4. Kinetics of hydrolysis with an immobilized enzyme

Kinetics of the reaction in the gel layer was determined in the conditions of convective flow of the substrate solution through the catalytic membrane with assumption the process runs in the kinetics regime. High value of the substrate stream in such a case allows us to assume that there is no substrate concentration gradient in the reaction layer (a differential reactor).

The reaction was carried out for different amounts of the enzyme mass immobilized on the membrane, i.e. different volumes of the reactor  $V_R$  [26], where the substrate solution in

the buffer of the volume ( $V_b$ ) was circulated through the membrane.

The proportion of the amount of substrate conversion to the reactor volume observed in experiments has confirmed rightness of the assumption of kinetic regime of the process. In these conditions:

$$r = \frac{dN_s}{V_R \cdot dt} = \frac{V_b}{V_R} \cdot \frac{dc_s}{dt} \quad (1)$$

The reaction rate was calculated on the basis of the measurements and is presented in Fig. 5. It was found that the kinetics of polypeptides

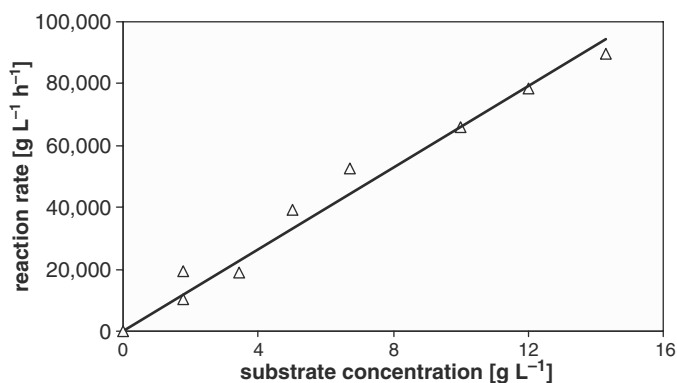


Fig. 5. Dependence of the reaction rate on the substrate concentration in reaction carried out by thermolysin adsorbed on the surface of nitrate cellulose membrane.

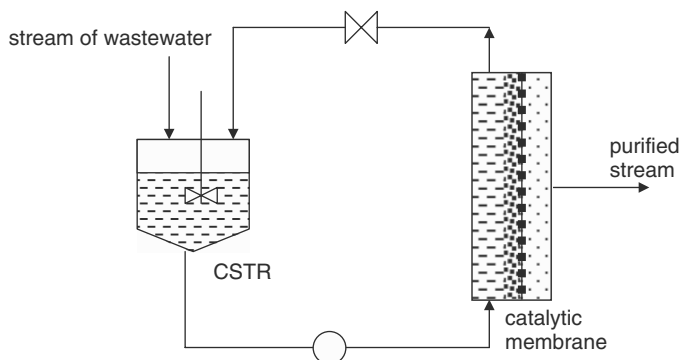


Fig. 6. Scheme of integrated system for peptides removing from wastewater stream in continuous process.

hydrolysis could be described in the applied range of substrate by the first-order equation:

$$r = k_r \cdot c_s \quad (2)$$

where the constant  $k_r$  for the tested reaction is equal  $6615.4 \text{ h}^{-1}$ .

When we recalculate the specific enzyme activity in its native and immobilized form a significant activity decrease of the immobilized form is observed. It is presumed that this is a result of large dimensions of substrate molecules (almost comparable to the molecules of enzyme) at the beginning of the process and consequently big difficulties in access of the substrate to the active centers of biocatalyst. It looks that immobilized enzyme will be more efficient in catalysis of much shorter chain of substrate molecules (there are some data confirmed this thesis), which occur at further stages of the hydrolysis (e.g. at  $\alpha > 0.5$ ). This creates an idea to integrate in series two bioreactors with native and next with immobilized enzyme.

#### 4. Conclusion

Immobilized thermolysin preparations obtained by adsorption on the surface of a cellulose nitrate membrane are characterized by high efficiency of protein binding with membrane surface, average

activity in relation to the initial substrate composed mainly of long polypeptide chains but a very low stability results most probably from a gradual detachment of the enzyme from the membrane.

When this type of reactions is applied to wastewater treatment it is important that (i) process runs fast, (ii) reactor volume is not so much extended, (iii) substrate concentration at the exit is very low (i.e. highly purified wastewater), (iv) process economic is satisfied. Because of these facts and having data concern the immobilized enzyme it is proposed that the total mass of biocatalyst used in the reaction be divided in a controlled way between two reaction zones operating in series (Fig. 6). Part of the enzyme mass will be maintained in the native form within the tank reactor volume while the other part will be deposited on the membrane surface due to adsorption and will constitute a reactor with a fixed bed of biocatalyst. The process of hydrolysis of initial substrate (polypeptides) will be carried out with high enzyme activity in CSTR and next the stream will be directed to the packed bed where the final hydrolysis will be performed. In the packed bed the substrate will be at low-molecular and at low concentration and the process normally slow in CSTR due to low substrate concentration will be fasted by high enzyme concentration in the layer immobilized on membrane.

The presence of a properly selected membrane causes that enzyme molecules cannot leave the bioreactor (important costs reduction), they can only transfer between the packed zone on the membrane surface and the zone of complete mixing in the reactor volume. This is a dynamic process and the method of control of the biocatalyst amount immobilization will be the main research goal of the authors.

Thickness of the deposited enzyme layer should be kept on a level sufficient to high substrate conversion in order that its concentration at the exit is close to zero (i.e. wastewater will be highly purified). The control of the layer thickness will be made by a proper selection of transmembrane pressure and turbulence of liquid flow tangential to the membrane. Stability of enzyme fixing to the membrane due to sorption is insignificant in this case because, by definition, part of the enzyme mass is in the native form in the circulating solution. Additionally, unstable fixing of the enzyme with the membrane will be used in the biocatalyst bed recovery in order to keep its required activity by dynamically creation enzyme layer during the whole time of the process.

Characteristics of the process performed in an integrated system (CSTR + catalytic membrane) will be a subject of further studies.

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### Nomenclature

$A$	membrane surface, $\text{cm}^2$
$c_s$	substrate concentration, $\text{g L}^{-1}$
$K_M$	constant of Michaelis–Menten, $\text{g L}^{-1}$
$k_r$	first-order reaction rate constant, $\text{h}^{-1}$
$k_3$	reaction rate constant, $\text{g}_{\text{pr.}} \cdot \text{g}_{\text{enz.}}^{-1} \text{h}^{-1}$
$N_s$	substrate amount, mol

$r$	reaction rate, $\text{g L}^{-1} \text{h}^{-1}$
$t$	time, s, h
$V_b$	substrate tank volume, L
$V_R$	reactor volume, L

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